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Oxidative radioiodination damage to human lactoferrin

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Oxidative iodination of human lactoferrin (Lf) as commonly performed by using the chloramine-T, the Iodogen or the lactoperoxidase method produces an unreliable tracer protein because of excessive and heterogeneous polymer formation. Before iodination a minor tetramer fraction may be demonstrable in iron-saturated Lf only. Iodination-induced polymerization of iron-poor as well as iron-saturated Lf occurs independently of the presence or absence of 10 mM-EDTA and the $^{125}I^-$/Lf molar ratio used for iodination. ^{125}I-Lf polymers are mainly covalently linked, as suggested by the lack of substantial dissociation in SDS/polyacrylamide-gel electrophoresis. Damage to the ^{125}I-Lf monomer may be another consequence of oxidative iodination. This is demonstrated in SDS/polyacrylamide-gel electrophoresis where 50% of the radioactivity of apparently normal monomer (M_r 75000) is displaced to a lower-M_r region (30000–67000) after reduction with dithiothreitol. Non-oxidative iodination by the Bolton–Hunter technique produces an antigenetically stable tracer that is not being subjected to polymerization and monomer degradation as judged by high-performance gel chromatography and SDS/polyacrylamide-gel electrophoresis with and without dithiothreitol treatment. It is concluded that oxidation in itself leads to covalent non-disulphide cross-linking between human Lf molecules and, possibly, to intramolecular peptide-bond breaking becoming unmasked under reducing conditions. In biological experiments with human ^{125}I-Lf this problem should be carefully considered.

INTRODUCTION

In its native form the iron-binding glycoprotein lactoferrin (Lf) appears to be involved in the pathogenesis of hyposideraemia of inflammation (Van Snick *et al.*, 1974), in granulocyte adherence (Boxer *et al.*, 1982) and bacteriocidy (Ambruso & Johnston, 1981) and in feedback inhibition of myelopoiesis (Broxmeyer, 1979; Bagby *et al.*, 1981). However, the understanding of the various biological functions of Lf depends on reliable methods for its immunoassay in plasma and for the study of its kinetics. Unfortunately, there is disagreement on radioimmunologically determined plasma concentrations in normal humans, ranging from approx. 1 mg/l (Bennett & Kokocinski, 1979) to approx. 10 μg/l (Broxmeyer *et al.*, 1983), and on kinetic data such as the initial half-life in human plasma of intravenously injected ^{125}I-Lf, ranging from approx. 2 min (Fillet *et al.*, 1978) to approx. 90 min (Bennett & Kokocinski, 1979).

Considering that even native Lf may undergo self-aggregation (Bennett *et al.*, 1981) and interact with proteins (Hekman, 1971), polysaccharides (Imber & Pizzo, 1983), and even DNA (Bennett *et al.*, 1983), one might expect additional problems after radioiodination. Several authors have, in fact, mentioned some 'immunodamage' (Rümke *et al.*, 1971), aggregation (Bennett & Mohla, 1976; Bennett & Kokocinski, 1979) and an altered electrophoretic mobility (Karle *et al.*, 1979) of radioiodinated Lf. Nevertheless, in many studies relying on iodinated Lf, the quality of ^{125}I-Lf was not further questioned, once it had been separated from $^{125}I^-$ by gel chromatography or anion-exchange chromatography.

In the present study we demonstrate that every method that is currently used for the iodination of Lf (chloramine-T, Iodogen and lactoperoxidase) produces uncontrollable alterations of the native protein and, hence, an unreliable tracer. It is further shown that the damage mainly results from formation of covalent complexes and, to some extent, denaturation. Comparing the effects of oxidative iodination procedures directly iodinating tyrosine residues of Lf with those of a non-oxidative method (Bolton–Hunter), by which a ^{125}I-labelled active ester is covalently coupled to amino groups of the protein, we demonstrate the susceptibility of human Lf to oxidative stress.

EXPERIMENTAL

Materials

For gel chromatography Sephadex G-25, Sephadex G-100, Superose 6 and the M_r standards from Pharmacia Fine Chemicals (Uppsala, Sweden) and for anion-exchange chromatography Dowex AG1-X8 from Bio-Rad Laboratories (Richmond, CA, U.S.A.) were used. Commercial human Lf and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The radioiodination reagents were: chloramine-T from BDH Chemicals (Poole, Dorset, U.K.); Iodogen from Pierce Chemicals (Rockford, IL, U.S.A.); Enzymobead Radioiodination Reagent from Bio-Rad Laboratories; Bolton–Hunter Reagent from Amersham International (Amersham, Bucks., U.K.).

Isolation and saturation with iron of human Lf

Human Lf was isolated from fresh breast milk by affinity chromatography on heparin–Sepharose as described by Bläckberg & Hernell (1980). The degree of

Abbreviation used: Lf, lactoferrin.

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iron saturation was determined from the protein/iron molar ratio, the protein content being calculated from the amount of nitrogen as determined by the Kjeldahl method and iron being assessed colorimetrically with bathophenanthroline as a colour indicator. Our native Lf was shown to be 7% saturated with iron. For some experiments Lf in 0.15 M-sodium phosphate buffer, pH 7.4, was fully saturated with $Fe(NH_4)_2(SO_4)_2$, excess iron being removed on a Sephadex G-25 desalting column.

Antiserum against isolated human Lf

Antibodies were raised in rabbits, and produced a sharp identical precipitation line with either isolated or commercial human Lf. The antiserum did not cross-react with other milk proteins or with human transferrin.

Chromatographic and electrophoretic procedures

The purity of native and of radiolabelled Lf was assessed by gel chromatography in 0.1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.4, in a high-performance Superose 6 gel column (1.6 cm × 62 cm) and by SDS/polyacrylamide-gel electrophoresis. In some instances SDS/polyacrylamide-gel electrophoresis was carried out after overnight reduction at room temperature with dithiothreitol of labelled protein in the presence of carrier Lf. The M_r standards for gel filtration were lysozyme (M_r 14500), plasminogen (M_r 90000), fructose-bisphosphate aldolase (M_r 158000), catalase (M_r 232000) and ferritin (M_r 450000). Phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000) and carbonic anhydrase (M_r 30000) were used as standards for polyacrylamide-gel electrophoresis. Isolated native Lf migrated as one band between bovine serum albumin and phosphorylase *b*.

Iodination techniques

Iodination of iron-poor and iron-saturated Lf with ^{125}I was performed by four techniques.

Chloramine-T method (Hunter & Greenwood, 1962). Lf (10 μl of a 1 mg/ml solution in 0.15 M-sodium phosphate buffer, pH 7.4) was treated with 0.5 mCi of carrier-free $^{125}I^-$ in 25 μl of 0.4 M-sodium phosphate buffer, pH 7.4, and with 10 μl of chloramine-T (2.5 mg/ml) for 15–20 s. The reaction was stopped by the addition of 100 μl of sodium metabisulphite (2.5 mg/ml) and 100 μl of KI (5 mg/ml). To separate labelled protein from remaining free $^{125}I^-$ the incubation mixture was passed through a 0.5 cm × 18 cm Sephadex G-100 column equilibrated with 0.15 M-Tris/HCl buffer, pH 7.4, containing 0.1% bovine serum albumin and, in some experiments additionally, through a small anion-exchange column (AG1-X8).

Iodogen method (Salacinski *et al.*, 1981). Conical polypropylene tubes containing 150 μl of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) in chloroform (0.25 mg/ml) were evaporated to dryness with N_2. Lf (10 μl of a 1 mg/ml solution in 0.15 M-sodium phosphate buffer, pH 7.4) and 0.25 or 0.5 mCi of $^{125}I^-$ in 10 μl of 0.4 M-sodium phosphate buffer, pH 7.4, were subsequently added to the tubes homogeneously coated with Iodogen. After a reaction time of 2 or 15 min respectively the reaction was stopped by the addition of 500 μl of 0.05 M-sodium phosphate buffer, pH 7.4, and by transferring the solution to another tube. The labelled Lf was then separated from iodide as above.

Solid-phase lactoperoxidase/glucose oxidase method (Burgett *et al.*, 1977). A suspension (25 μl) of Enzymobead radioiodination reagent was used to iodinate Lf (10 μl of a 1 mg/ml solution in 0.15 M-sodium phosphate buffer, pH 7.4) with 1 mCi of carrier-free $^{125}I^-$ in 25 μl of 0.4 M-sodium phosphate buffer, pH 7.4, in the presence of 25 μl of 1% (w/v) D-glucose. After 30 min the reaction was stopped by the addition of 500 μl of 0.15 M-sodium phosphate buffer, pH 7.4, containing 0.05% NaN_3. The reaction mixture was then centrifuged at 1000 *g* for 5 min, and the labelled Lf was separated from iodide as above.

Iodination with the Bolton–Hunter reagent. The original description (Bolton & Hunter, 1973) was modified in that Lf (10 μl of a 0.5 mg/ml solution) was iodinated in 0.15 M-sodium phosphate buffer, pH 7.4, the protein being incubated for 1 h at 0 °C and then for 18 h at 4 °C with the evaporated *N*-hydroxysuccinimide ester containing 0.25 mCi of ^{125}I. The iodinated protein was recovered from the reaction vial with 400 μl of 0.15 M-sodium phosphate buffer, pH 7.4, and separated from iodinated hydrolysis products by gel filtration and/or anion-exchange chromatography. The content of free iodide in unpurified and purified radiolabelled protein was measured by t.l.c.

RESULTS

Adsorption on Sephadex and antibody binding of ^{125}I-Lf

Oxidative iodination. Oxidative iodination by the chloramine-T, Iodogen and lactoperoxidase methods produced ^{125}I-Lf tracer exhibiting an abnormally high adsorption on Sephadex and an early loss of antibody-binding capacity (Table 1).

During the separation of ^{125}I-Lf from free $^{125}I^-$ 75–85% of the total radioactivity was adsorbed on the entire Sephadex G-100 gel presaturated with bovine serum albumin. Of this, 55–70% was protein-bound, as shown in SDS/polyacrylamide-gel electrophoresis after elution of the adsorbed radioactivity in 0.1% SDS. In view of the formation of such massive amounts of non-viable tracer, a reliable estimation of the iodination efficiency or the specific radioactivity of ^{125}I-Lf cannot be assumed. Lf is also subjected to considerable non-covalent binding of radioactive iodide. This was demonstrated with the Iodogen method, where almost 50% of the protein-associated radioactivity could be removed from Lf by incubation of the tracer solution with non-radioactive iodide.

Antibody binding of oxidatively labelled ^{125}I-Lf was adequate for a short period only, decreasing by at least 50% within 4 weeks.

Non-oxidative iodination. Non-oxidative iodination by the Bolton–Hunter method resulted in low adsorption on Sephadex G-100 and adequately stable antibody binding of ^{125}I-Lf (Table 1).

Formation of ^{125}I-Lf complexes

In order to define the protein alterations induced by radioiodination more precisely, high-performance gel

Table 1. Iodination conditions, tracer recovery and antibody binding of ^{125}I-Lf

For experimental details see the text.

Iodination method	$^{125}I^-$/Lf molar ratio	Iodination time	Tracer purification on Sephadex G-100: Adsorption of ^{125}I radioactivity (%)*	Tracer purification on Sephadex G-100: Elution of ^{125}I-Lf (%)†	Antibody-binding (%)‡: Initial	Antibody-binding (%)‡: 4 weeks after iodination
Chloramine-T	2.2	20 s	81	7	53	10
Lactoperoxidase	2.2	30 min	83	2	30	15
Iodogen	1.1	15 min	75	15	40	20
	1.1	2 min	86	9	—	—
Bolton–Hunter	—	1 h	10	15	38	30

* The adsorbed radioactivity was completely eluted from the Sephadex G-100 column with 0.1% SDS. Of this, 55–70% was identified on polyacrylamide-gel electrophoresis as protein-bound radioactivity.

† Irrespective of the iodination technique ^{125}I-Lf was eluted in one sharp and homogeneous protein peak. The difference from 100% (total radioactivity applied to the column) of the adsorbed radioactivity plus the eluted ^{125}I radioactivity is accounted for by free $^{125}I^-$ (oxidative iodination) or iodinated hydrolysis products (Bolton–Hunter) eluted from Sephadex G-100.

‡ Antibody binding ($B_0 = B/T$) was determined at a 1:60000 antiserum dilution.

chromatography was carried out on native, non-oxidatively labelled (Bolton–Hunter method) and oxidatively labelled (Iodogen method) Lf.

Native Lf. Unlabelled Lf was quantitatively eluted from Superose 6 in a sharp peak at M_r 75000. This peak proved to be highly reproducible for iron-poor as well as for iron-saturated Lf if the protein load did not exceed 1 mg. If 4 mg of iron-saturated Lf was applied to the column, an additional fraction comprising less than 10% of the total protein load was eluted at an M_r of approx. 300000 (Fig. 1*a*), suggesting the presence of Lf polymers. These polymers could not be dissociated by 10 mM-EDTA and were only observed with iron-saturated Lf.

Non-oxidative iodination. After non-oxidative iodination and Sephadex G-100 gel filtration the elution profile on Superose 6 of ^{125}I-Lf was not substantially different from that of native Lf (Fig. 1*b*). Again, a small amount of polymers was demonstrable only in iron-saturated ^{125}I-Lf.

Oxidative iodination. Iodogen iodination resulted in a marked heterogeneity of ^{125}I-Lf. The apparently homogeneous protein peak recovered from the Sephadex G-100 column, used to separate labelled protein from free iodide, was shown to consist of high-M_r complexes of iron-saturated (Fig. 2*a*) and iron-poor (Fig. 3*a*) ^{125}I-Lf. The main peaks were situated in the dimer and hexamer regions and were only partially removed by subsequent anion-exchange chromatography (Figs. 2*b* and 3*b*). A consistent displacement of the 'monomer' peak from M_r 75000 to M_r approx. 90000–120000 persisting in aging tracer was also observed (Fig. 4). With increasing age of the tracer, however, less ^{125}I-Lf complexes were found in solution, the decrease in radioactivity being due to an increased adsorption of the tracer on the storage vial.

Formation of ^{125}I-Lf complexes after Iodogen, chloramine-T and lactoperoxidase iodination was also confirmed if only low amounts of $^{125}I^-$ (< 0.5 mol of

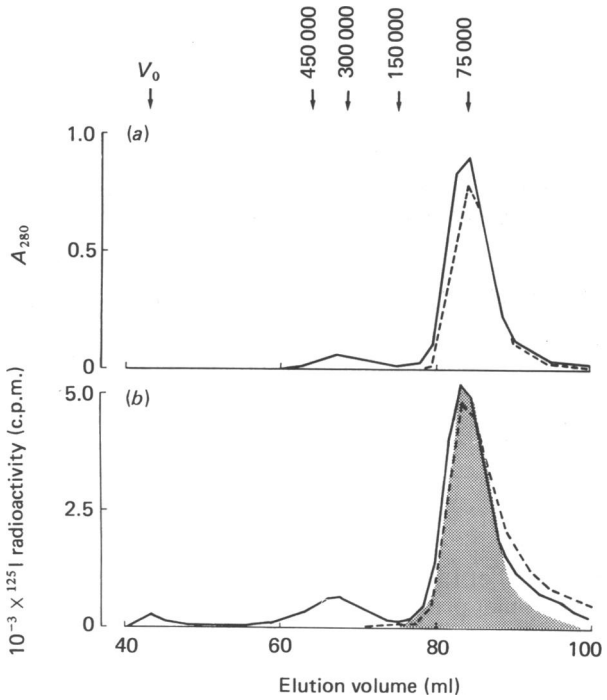

Fig. 1. Gel chromatography of native Lf and after iodination under non-oxidative conditions

Gel chromatography was performed on Superose 6 [1.6 cm × 62 cm; V_0 (void volume) = 42 ml] in 0.15 M-Tris buffer, pH 7.4. (*a*) Elution profile of native iron-poor (----) and iron-saturated (——) Lf (total protein load 4 mg). (*b*) Elution profile of iron-poor (----) and iron-saturated (——) ^{125}I-Lf produced by non-oxidative iodination (Bolton–Hunter method) after Sephadex G-100 gel filtration for removal of unbound iodide. Gel chromatography of ^{125}I-Lf was always performed in the presence of 1 mg of native Lf as a carrier protein and a marker for normal Lf monomer elution. The elution profile of native Lf, being highly reproducible, is represented as an identical stippled area in this and subsequent Figures. Also, the positions of M_r markers are indicated at the top of this and subsequent Figures.

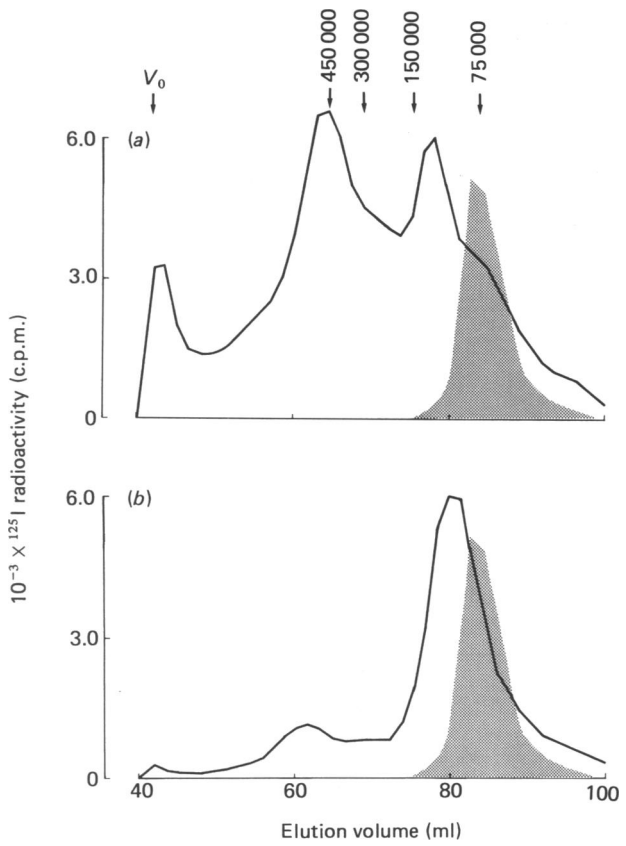

Fig. 2. Gel chromatography of iron-saturated Lf after iodination under oxidative conditions

Gel chromatography was peformed on Superose 6 (1.6 cm × 62 cm; $V_0 = 42$ ml) in 0.15 M-Tris buffer, pH 7.4. (*a*) Elution profile of iron-saturated oxidatively iodinated ^{125}I-Lf (Iodogen method followed by Sephadex G-100 gel filtration). (*b*) Elution profile of the same ^{125}I-Lf sample after an additional purification step on an AG1-X8 anion-exchange column. For carrier protein and stippled area see Fig. 1 legend.

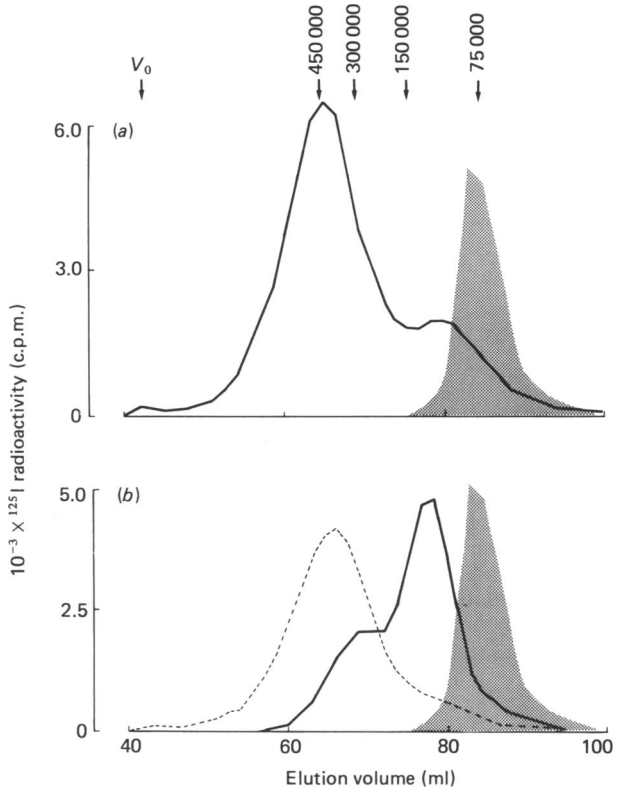

Fig. 3. Gel chromatography of iron-poor Lf after iodination under oxidative conditions

Gel chromatography on Superose 6 (1.6 cm × 62 cm; $V_0 = 42$ ml) in 0.15 M-Tris buffer, pH 7.4. (*a*) Elution profile of iron-poor oxidatively iodinated ^{125}I-Lf (Iodogen method, followed by Sephadex G-100 gel filtration). (*b*) Elution profile of the same ^{125}I-Lf sample after an additional purification step on an AG1-X8 anion-exchange column. The broken line represents ^{125}I radioactivity that subsequently could be eluted from AG1-X8 by 1 M-NaCl. For carrier protein and stippled area see Fig. 1 legend.

iodide/mol of Lf) were used (Fig. 5). No displacement of the monomer peak, however, was observed at low-iodide iodination.

Stability and degradation of ^{125}I-Lf complexes

A polymer fraction (M_r approx. 300000) and the monomer (M_r 75000) of iron-saturated oxidatively and non-oxidatively iodinated ^{125}I-Lf preparations, identified on Superose 6, were subjected to polyacrylamide-gel electrophoresis under denaturing (SDS) and reducing (SDS + dithiothreitol) conditions. Gels were cut such as to identify monomers (M_r 75000), non-dissociated or incompletely dissociated aggregates ($M_r < 94000$) and degradation products (M_r 30000–67000) (Table 2).

Polymer fraction (M_r approx. 300000). In 1% SDS only 30% of either polymer species dissociated into monomers, and no further dissociation could be accomplished by reduction with dithiothreitol. This indicates that covalent intermolecular bonds other than disulphides had been formed during the polymerizing process.

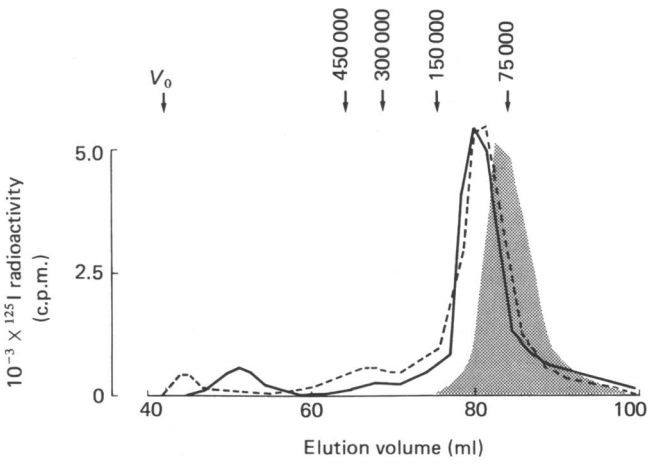

Fig. 4. Gel chromatography of iron-saturated Lf iodinated under oxidative conditions: effects of storage of tracer

Gel chromatography was performed on Superose 6 (1.6 cm × 62 cm; $V_0 = 42$ ml) in 0.15 M-Tris buffer, pH 7.4. Elution profile of iron-saturated oxidatively iodinated ^{125}I-Lf, 4 (——) and 10 (– – – –) weeks after iodination and storage at 4 °C. For carrier protein and stippled area see Fig. 1 legend.

Table 2. Electrophoretic analysis of gel-chromatography fractions (Figs. 1 and 2)

SDS (1%)/polyacrylamide-gel electrophoresis (T = 5%, C = 4%) of a polymer fraction (M_r 300000) and monomer (M_r 75000) of non-oxidatively (Bolton–Hunter method) and oxidatively (Iodogen method) iodinated iron-saturated ^{125}I-Lf. The values in parentheses give the percentage radioactivities recovered in the same fractions after reduction with dithiothreitol. Electrophoresis of the respective Superose 6 gel-chromatography fractions of ^{125}I-Lf was carried out in the presence of native Lf (10 μg) used as a reference for Lf monomer migration. Gels were stained with Coomassie Blue and divided into high-M_r (M_r < 94000), monomer (M_r 75000) and low-M_r (M_r 30000–67000) sections for determination of radioactivity.

Gel-chromatography fraction	SDS/polyacrylamide-gel electrophoresis (% radioactivity)		
	M_r > 94000	M_r 75000	M_r < 67000
Non-oxidative iodination			
M_r 300000	64 (63)	30 (27)	6 (10)
M_r 75000	11 (15)	86 (79)	3 (6)
Oxidative iodination			
M_r 300000	58 (53)	33 (16)	9 (31)
M_r 75000	29 (34)	59 (28)	12 (38)

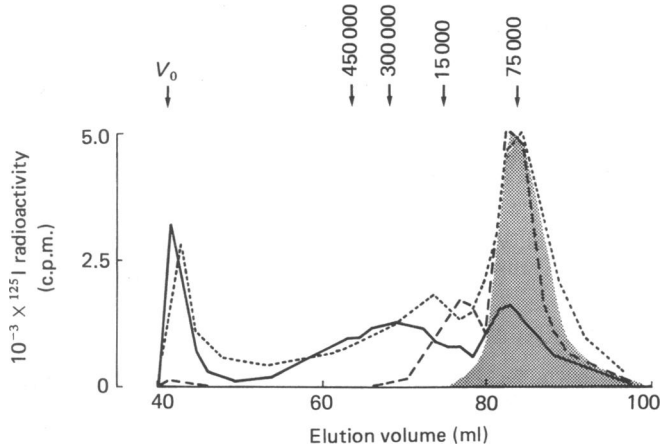

Fig. 5. Gel chromatography of iron-poor Lf iodinated under oxidative conditions with low iodide/protein ratios

Gel chromatography was performed on Superose 6 (1.6 cm × 62 cm; V_0 = 42 ml) in 0.15 M-Tris buffer, pH 7.4. Elution profile of iron-poor ^{125}I-Lf after oxidative iodination with a low amount of ^{125}I$^-$ (< 0.5 mol of iodide/mol of Lf) and subsequent Sephadex G-100 gel filtration. Iodination methods: chloramine-T (------), Iodogen (– – – –) and lactoperoxidase (——). For carrier protein and stippled area see Fig. 1 legend.

Monomer fraction (M_r 75000). In SDS almost 90% of non-oxidatively labelled ^{125}I-Lf monomers co-migrated with native Lf. This was in contrast with only 60% of oxidatively iodinated ^{125}I-Lf monomers co-migrating with unlabelled Lf. The main difference between the two types of monomer, however, consisted in their respective response to reduction: whereas non-oxidatively labelled ^{125}I-Lf monomers were virtually unaffected by dithiothreitol, less than 50% of oxidatively labelled ^{125}I-Lf monomers were recovered at their original position after reduction. Since most of the 'displaced' radioactivity was recovered in the low-M_r section of the gel (M_r 30000–67000), some protein degradation becoming unmasked by reductive treatment has to be assumed.

DISCUSSION

For radioimmunoassays or biological studies Lf is commonly radioiodinated. The most widely used reagents, chloramine-T, Iodogen and peroxidases, all act as oxidants transforming the iodide into a I^0 or I^+ state, which then attacks the tyrosine residues of the protein to form either monoiodinated or di-iodinated tyrosine (Huwiler *et al.*, 1985). A few observations (Rümke *et al.*, 1971; Hansen *et al.*, 1975; Bennett & Mohla, 1976) have suggested that human Lf might be structurally altered by radioiodination. Surprisingly, however, the purity and stability of ^{125}I-Lf widely being used as a tracer substance were usually not questioned, once it had been separated from free iodide and a satisfactory specific radioactivity and antibody-binding capacity had been established.

In order to iodinate human Lf isolated from fresh human breast milk we used limiting ^{125}I$^-$ concentrations (1.1–2.2 mol of ^{125}I$^-$/mol of Lf), short iodination times and current oxidative iodination techniques (chloramine-T, Iodogen, lactoperoxidase). After iodination, apparently homogeneous ^{125}I-Lf was eluted from Sephadex G-100 used for the separation of free iodide. However, substantial ^{125}I-Lf adsorption on the Sephadex gel and non-covalent iodide binding to Lf was noted. Furthermore, a rapid deterioration of the antibody-binding capacity of ^{125}I-Lf, not accounted for by relevant deiodination, was observed. These indications of an impaired tracer quality after oxidative iodination and their virtual absence after non-oxidative iodination (Bolton–Hunter method) suggested a particular susceptibility of human Lf to oxidative conditions.

This question was further explored by high-performance gel chromatography. After oxidative iodination a massive amount of complexes of ^{125}I-Lf of the size of dimers up to hexamers was detected in the apparently homogeneous ^{125}I-Lf peak eluted from the Sephadex G-100 column. It should be emphasized that the polymers were formed irrespectively of the degree of iron saturation, the presence or absence of 10 mM-EDTA, the iodination technique and the concentration of ^{125}I$^-$ present in the iodination mixture. The iodide concentration did, however, appear to affect the structure of Lf

monomer: at commonly used iodide concentrations (1.1–2.2 mol of iodide/mol of protein) a displacement of the monomer peak towards a slightly higher-M_r region was reproducibly observed, which did not occur at very low iodide concentrations (< 0.5 mol of iodide/mol of Lf). In aging ^{125}I-Lf tracer an increasing shift of the radioactivity of soluble polymer to the wall of the storage vial and, if chromatography was repeated, to the top of the Superose gel was observed. We therefore believe that Lf polymerization is initiated by oxidative iodination and continues with time towards the formation of very-high-M_r complexes becoming increasingly insoluble and perhaps adhesive.

Non-oxidatively iodinated Lf did not exhibit the impressive heterogeneity of the oxidatively labelled compound. This is in agreement with its non-adhesiveness to Sephadex and its stable antibody-binding capacity. The Superose 6 elution profile and the pattern of radioactivity on polyacrylamide-gel electrophoresis were virtually identical with that of native, iron-poor (one peak at M_r 75000) and iron-saturated (additional minor tetramer peak) Lf.

The reproducibility of polymer formation under different oxidative but not under non-oxidative conditions, and the fact that polymers were formed excessively at the moment of iodination and did not depend on the amount of radioactive iodide present, suggested a causative role of oxidation in itself.

In order to determine the type of intermolecular linkage, two corresponding gel-chromatography fractions (M_r 300000 and 75000) of oxidatively and non-oxidatively iodinated Lf were subjected to SDS/polyacrylamide-gel electrophoresis with and without prior reduction with dithiothreitol. Our results suggest that the major part of pre-existing Lf polymers (in iron-saturated Lf) as well as Lf polymers induced by oxidative iodination are linked by covalent non-disulphide bonds, since only 30% of either species were disrupted by dithiothreitol treatment. Oxidatively induced covalent intermolecular bonds have previously been reported to form between several proteins in the presence of lactoperoxidase (Aeschbach *et al.*, 1976) and between chloramine-T-treated epidermal growth factor and its receptor (Comens *et al.*, 1982). It is also known that oxidative iodination may alter amino acid residues such as methionine and cysteine (Wood *et al.*, 1981) and histidine (Sjöholm & Sjödin, 1974) in different proteins, or even produce a cleavage of peptide bonds such as at tryptophan (Alexander, 1974) and tyrosine (Junek *et al.*, 1969). Our finding that after incubation with dithiothreitol roughly 50% of the radioactivity of oxidatively iodinated Lf monomers migrated ahead of the carrier Lf (M_r < 75000) in SDS/polyacrylamide-gel electrophoresis suggests that the oxidative conditions under which iodinations are accomplished might, in fact, induce the breaking of one or two peptide bonds in human Lf too. Complete disruption would then ensue, when, in addition, the disulphide bonds are broken up by a reducing agent such as dithiothreitol.

In conclusion, we propose that oxidation, such as produced by most conventional iodination techniques, leads to a mainly covalent non-disulphide-bond polymerization of human Lf, which might, to some extent, be the consequence of a peptide-bond breaking causing an alteration of the tertiary structure of the molecule and which may escape detection by usual gel-chromatography procedures. Apart from the general problem of a potentially damaging effect by oxidative iodination on diverse protein structures, as outlined above, it is noteworthy that very similar degradation and aggregation processes as observed in Lf have been documented after iodination with chloramine-T, Iodogen and lactoperoxidase of human spleen ferritin (Bolton *et al.*, 1979) and prostatic acid phosphatase (McCarthy & Markowitz, 1983; Sturgeon *et al.*, 1983). As in the case of human Lf, the protein alterations were not due to overiodination and could be circumvented by a non-oxidative iodination technique. Since Lf as well as ferritin and acid phosphatase (Keough *et al.*, 1980) are iron-binding proteins, a mediator role of iron for the particular susceptibility of these proteins to oxidative stress might be envisaged, although to our knowledge pertinent data on human serum transferrin are lacking to date.

From our experience with iodinated human Lf we consider it crucial that the iodinated protein must be checked for homogeneity and stability with exceptional care, before its molecular integrity and, hence, its representative role in biological systems is taken for granted. Together with the observation by others of a polymerization-induced functional impairment of human Lf (Bagby & Bennett, 1982), our results suggest that previous reports on quantitative aspects of ^{125}I-Lf–cell interactions *in vitro* (Van Snick & Masson, 1976; Birgens *et al.*, 1983) and *in vivo* (Imber & Pizzo, 1983; Regoeczi *et al.*, 1985) may have to be challenged.

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